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Form Approved
OMB No. 0704-0188

1. [illegible] (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
		Reprint

4. TITLE AND SUBTITLE

Title shown on Reprint

5. FUNDING NUMBERS

DAAL03-88-K-0196

6. AUTHOR(S)

Authors listed on Reprint

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Univ of Medicine & Dentistry of NJ
Piscataway, NJ 08854-5635

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U. S. Army Research Office
P. O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

ARO 24931.7-LS

11. SUPPLEMENTARY NOTES

The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

ABSTRACT SHOWN ON REPRINT

14. SUBJECT TERMS	15. NUMBER OF PAGES
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

Michael J. Leibowitz, Francis P. Barbone, and Denise E. Georgopoulos, Factors determining translational efficiency of mRNA in yeast, Biotechnology-Biodegradation Workshop, U.S. Army Research Office, Cashiers, NC, pp.66-73 (1990).

Factors Determining Translational Efficiency of mRNA in Yeast

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Killer virus of Saccharomyces cerevisiae is a cytoplasmically-inherited virus that confers on persistently-infected yeast cells the ability to secrete a protein toxin which kills uninfected yeast cells but to which infected cells, denoted killers, are resistant (reviewed by Tipper and Bostian, 1984; Wickner, 1986). Killer toxin production can be assayed by halos of killing of a lawn of uninfected cells around killer colonies on a petri dish. The genome of the virus consists of two segments of double-stranded (ds) RNA that are separately encapsidated in cytoplasmic virions localized in the cytoplasm of infected cells. The L-A dsRNA segment is 4.9 kbp in length and encodes the major capsid protein (81 kd) of the virions and possibly a larger capsid-RNA polymerase fusion product by a -1 frame shift occurring at the translational level, analogous to that producing retrovirus gag-pol fusion protein (Icho and Wickner, 1988; Diamond et al, 1989). The M dsRNA segment (1.8 kbp) is dependent on L-A dsRNA for its replication and is responsible for the killer and resistance phenotypes of cells infected with both dsRNA segments. The positive strand of the M dsRNA molecule encodes the 32 kd preprotoxin precursor of toxin and resistance substance on its 5' proximal 1000 bases, followed by a 200 base internal polyA tract and 630 bases without any extensive open reading frames (Hannig et al, 1984; Skipper et al, 1984; Georgopoulos et al, 1986).

Gene expression by killer virus can be studied in vitro. Virions isolated from infected cells contain RNA polymerase activity which produces full-length positive polarity copies of the M and L-A dsRNA segments, denoted m and l, respectively (Hannig et al, 1984; Thiele et al, 1984). These transcripts, in turn, encode the viral polypeptide gene products when added to a cell-free translational extract of yeast (Hussain et al, 1986; Leibowitz et al, 1990). The m transcript, unlike l and many other mRNAs, has a 64 nucleotide hairpin

loop at its 5' terminus with the initiating AUG of the preprotoxin open reading frame (nucleotides 14-16) in the 5' portion of the stem and loop structure, with nearby sites of potential complementarity to the 3' terminal regions of 18S and 5.8S rRNAs (Leibowitz et al, 1983; Hannig et al, 1984). These structural features may indicate that translational initiation on m transcript may occur by a mechanism distinct from that of other mRNAs lacking these features. The existence of different classes of mRNA molecules having subtle differences in their translation initiation mechanisms provides a potential site at which translational regulation, which appears to be important in eukaryotic cells, might take place. Since killer virus preprotoxin is readily expressed from cDNA clones (Skipper et al, 1984), site-directed mutagenesis of such clones should determine how the structural features at the 5' terminus of m transcript affect its translatability. This information will be used to design a vector optimizing translational gene expression in yeast. If the problem of regeneration of viral RNA from cDNA can be solved, further amplification should be possible since infected cells can harbor 1000 or more copies of viral dsRNA molecules per cell (Wickner, 1986), far surpassing copy numbers of any DNA vectors of yeast.

Vector Construction

In order to better quantitate the effect of mRNA 5' structure on translation we designed a vector with fewer RNA start sites than present in most nuclear genes of yeast, which should express the 5' terminus of m-derived cDNA relatively faithfully. Also, we created a fusion of the 5' portion of m-derived cDNA with the lacZ open reading frame, so that polypeptide expression in vivo could be readily quantitated by assaying β-galactosidase activity rather than the relatively non-quantitative measurement of toxin. This fusion has been

cloned adjacent to the CUP1 (metallothionein) promoter of yeast, which is copper-inducible and initiates only at two closely spaced sites, with both transcripts beginning with the sequence GAA... (Karin et al, 1984). The construct has been made in a yeast-E. coli shuttle vector, with the m'/'lacZ fusion linked to the promoter of CUP1 so that the m' 5'-terminal sequence GAA... replaces the homologous sequence at the downstream start site of the promoter.

In order to test the effect of 5' terminal mRNA sequences on translation in vitro, a parallel construction has been made with M-derived cDNA ligated into the SmaI site of plasmid pPM1 (Ahlquist and Janda, 1984). Such constructs can be transcribed in vitro by E. coli RNA polymerase to yield transcripts with authentic 5' termini and 3' termini several bases longer than the 3' end of the inserted cDNA. We are now testing polymerase chain reaction amplified fragments of our construct as templates for E. coli RNA polymerase in order to produce RNA products with both 5' and 3' termini colinear with the cDNA insert. These transcripts and similar transcripts of mutagenized plasmids will be tested as translational templates in a yeast cell-free translation system (Hussain and Leibowitz, 1986; Leibowitz et al, 1990). Thus we plan to test the effect of RNA structure on translational expression in yeast in vivo and in vitro.

Utilization of Exogenous Caps in Viral Transcription

The translational efficiency of mRNA in eukaryotic cells depends not only on its 5' terminal sequence but also on the presence of 5' terminal cap structures. The cytoplasmic location of killer virions in infected yeast cells (Dihanich et al, 1989) indicates that they face a problem which must be solved by many cytoplasmic viruses: how to translate viral messages in a cytoplasm which

prefers to translate messages containing 5'-terminal caps but which lacks host capping enzymes that are located in the nucleus (Itoh et al, 1987). The structure at the 5' terminus of viral transcripts in vivo is unknown; there is no evidence that genomic dsRNA or transcripts produced in vitro by the virion-associated RNA polymerase contain 5'-terminal cap structures (Bruenn and Keitz, 1976; Hannig et al, 1984; Thiele et al, 1984). However, yeast host cell mRNAs have 5'terminal type "0" caps, with the structure ^{7}mGpppXp--- (Spripati et al, 1976).

We have found that m and l transcripts produced by the killer virion-associated RNA polymerase in the presence of ^{7}mGpppG (500 μM) are at least 10 times as active as translational templates in the yeast cell-free protein synthesis system as are transcripts made in the absence of exogenous caps. Bacterial and bacteriophage DNA-dependent RNA polymerases are known to utilize ^{7}mGpppG as a primer for RNA synthesis, thus producing capped transcripts without post-transcriptional guanylylation. However, we found that m and l transcripts produced by the viral RNA polymerase reaction in the presence of ^{7}mGpppG were not immune precipitable by antibody to ^{7}mG (Munns et al, 1982), unlike transcripts of various recombinant plasmids transcribed in the presence of ^{7}mGpppG by the RNA polymerases of bacteriophage T7, bacteriophage SP6 or of E. coli.

The failure of viral m transcripts produced in the presence of ^{7}mGpppG to bind to antibody to ^{7}mG may not be due to 5' terminal secondary structure of m, since viral l transcripts produced in the presence of ^{7}mGpppG showed similar enhancement of their translational template activity and lack of antibody binding. Also, a 20-mer oligodeoxyribonucleotide hybridizing to the 5' terminus of a capped transcript of influenza virus cDNA produced in the presence of

^{7}mGpppG by bacteriophage T7 RNA polymerase failed to block its immune precipitability by anti-^{7}mG.

Plasmid pLS1 is a derivative of pPM1 into which cDNA derived from M dsRNA (with the polyA·polyU tract replaced by a short DNA segment containing an EcoRI site) is inserted at the SmaI site. When this plasmid is cut by EcoRI and transcribed by E. coli RNA polymerase, the expected 1100 base transcript is produced which should have the same 5' terminal sequence as m transcript produced by killer virus RNA polymerase. The authenticity of this transcript is indicated by its ability to direct synthesis of a product with identical molecular weight to preprotoxin (32 kd) in a yeast cell-free translation extract. When E. coli RNA polymerase is used to transcribe EcoRI-cut pLS1 in the presence of added ^{7}mGpppG, the resultant transcript is the same size as transcript made without cap, shows increased translational template activity in the yeast system, and is precipitable by antibody against ^{7}mG. Therefore, the failure of viral m transcript produced in the presence of ^{7}mGpppG to immune precipitate is not due to the subterminal sequence of the RNA, and seems to relate to its origin in the viral transcription process. Purified killer virions lack detectable RNA capping activities. The virions also have no activities altering the antigenicity of capped RNAs, since purified transcripts of pLS1 by E. coli RNA polymerase or of influenza NS gene cDNA by bacteriophage SP6 RNA polymerase remain intact and immune precipitable following incubation in a virion transcription reaction.

Therefore, it appears that killer virion-associated DNA-independent RNA polymerase utilizes ^{7}mGpppG as a primer for transcription in a different manner than this primer is utilized by various DNA-dependent RNA polymerases. Although the structure of the resultant product is unknown, one possibility is that viral

RNA polymerase may incorporate added caps into the structure 5'-Gppp^{7}mGp---, which may be antigenically distinct from the type "0" cap produced in ^{7}mGpppG-primed transcription by the other polymerases with the transcript structure 5'-^{7}mGpppGp---. The possible <u>in vivo</u> function of the unusual utilization of exogenous caps by killer virus remains unknown. However, such a mechanism might be part of the strategy by which the virus expresses its genetic information in a cap-dependent host cell cytoplasm.

ACKNOWLEDGMENT

This work was supported in part by contract 24931-LS from the U.S. Army Research Office. F.P. Barbone was a predoctoral fellow supported by Public Health Service Institutional Research Service Award CA-09069 from the National Cancer Institute. We thank P. Husak, R.W. Schlesinger, and P. Ahlquist for gifts of plasmids, and T. Munns for antiserum against ^{7}mG. Oligonucleotides used in this work were produced by L. Flores using an automated DNA synthesizer (ABI, Model 380B) in the DNA Synthesis Network Laboratory of the Center for Advanced Biotechnology and Medicine.

REFERENCES

Ahlquist,P., and M.Janda. 1984. Mol. Cell.Biol. 4:2876-2882.

Bruenn, J., and B. Keitz. 1976. Nucleic Acids Res. 3:2427-2436.

Diamond,M.E., J.J. Dowhanick, M.E. Nemeroff, D.F. Petras, C.Tu and J.A. Bruenn. 1989. J. Virol. 63:2983-3990.

Dihanich, M., E. Van Tuenen, J.D. Lambris and B. Marshallsay. 1989. Mol. Cell. Biol. 9:1100-1108.

Georgopoulos, D.E., E.M. Hannig and M.J. Leibowitz. 1986. In R.B. Wickner, A. Hinnebusch, L. Mets, A. Lambowitz, I.C. Gunsalus and A. Hollaender (editors), Extrachromosomal Elements in Lower Eukaryotes, Plenum, New York, pp. 203-214.

Hannig, E.M., D.J. Thiele,and M.J. Leibowitz. 1984. Mol. Cell. Biol. 4:101-109.

Hussain, I., and M.J. Leibowitz. 1986. Gene 46:13-23.

Icho, T., and R.B. Wickner. 1989. J. Biol. Chem. 264:6716-6723.

Itoh, H., H. Yamada, Y. Kaziro, and K. Mizumoto. 1987. J. Biol. Chem. 262:1989-1995.

Karin, M., R. Najarian, A. Halinger, P. Valenzuela, J. Welch and S. Fogel. 1984. Proc. Natl. Acad. Sci. USA 81:337-341.

Leibowitz, M.J., F.P. Barbone and D.E. Georgopoulos. 1990. Methods Enzymol. 194: in press.

Leibowitz, M.J., D.J. Thiele, and E.M. Hannig. 1983. In D.H.L. Bishop and R.W. Compans (editors), Double-stranded RNA Viruses, Elsevier/North-Holland Publishing Co., New York, pp. 457-462.

Munns, T.W., M.K. Liszewski, J.T. Tellam, H.F. Sims and R.E. Rhoads. 1982. Biochemistry 21:2922-2928.

Skipper N., D.Y. Thomas, and P. Lau. 1984. EMBO J. 3:107-111.

Sripati, C.E., Y. Groner and J.R. Warner. 1976. J. Biol. Chem. 251:2898-2904.

Thiele, D.J., E.M. Hannig and M.J. Leibowitz. 1984. Mol. Cell. Biol. 4:12-100.

Tipper, D.J., and K.A. Bostian. 1984. Microbiol. Rev. 48:125-156.

Wickner, R.B. 1986. Ann. Rev. Biochem. 55:373-395.